The cystathionine γ-lyase/hydrogen sulfide pathway mediates the trimetazidine-induced protection of H9c2 cells against hypoxia/ reoxygenation-induced apoptosis and oxidative stress

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Abstract

Objective: Trimetazidine is a piperazine-derived metabolic agent. It exerts cardioprotective effects against myocardial ischemia/reperfusion (I/R) injury. In addition, studies confirm that the cystathionine γ-lyase (CSE)/hydrogen sulfide (H_2S) pathway serves a beneficent role in attenuating myocardial I/R injury. However, the underlying role of the CSE/H_2S pathway in the trimetazidine-induced protection against myocardial I/R injury remains elusive. Therefore, this study investigated whether trimetazidine ameliorates hypoxia/reoxygenation (H/R)-induced H9c2 cardiomyocyte injuries in an *in vitro* cell model of myocardial I/R injury, by enhancing the CSE/H_2S pathway.
Methods: The H9c2 cell viability was determined with a cell counting Kit-8.
Results: Trimetazidine significantly increased the cell viability and decreased lactate dehydrogenase (LDH) release in H/R-treated H9c2 cells. Additionally, trimetazidine increased the H_2S levels and the CSE mRNA and protein levels, promoting the CSE/H_2S pathway under H/R conditions. The inhibition of the CSE/H_2S pathway, induced by transfection with specific siRNA against human CSE (si-CSE), eliminated the trimetazidine-induced upregulation of cell viability, downregulation of LDH release, increase of caspase-3 activity and apoptosis regulator BAX expression, and the decrease of apoptosis regulator Bcl-2 expression, which suggests involvement of the CSE/H_2S pathway in trimetazidine-induced cardioprotection. Furthermore, trimetazidine mitigated the H/R-induced increase in reactive oxygen species production and NADPH oxidase 2 expression, and decrease in superoxide dismutase activity and glutathione level, in H9c2 cells. These effects were also reversed by si-CSE.
Conclusion: This study revealed that the CSE/H_2S pathway mediates the trimetazidine-induced protection of H9c2 cardiomyocytes against H/R-induced damage by inhibiting apoptosis and oxidative stress. *(Anatol J Cardiol 2019; 22: 102-11)*
Keywords: trimetazidine, myocardial ischemia/reperfusion injury, cystathionine γ-lyase/hydrogen sulfide pathway, apoptosis, oxidative stress

Introduction

Myocardial ischemia/reperfusion (I/R) injury is associated with adverse cardiovascular outcomes following cardiac surgery, circulatory arrest or myocardial ischemia. It is one of the major causes of morbidity and mortality threatening human health (1, 2). Although apoptosis cascades, oxidative stress, mitochondrial dysfunction, and inflammation are recognized as the key drivers for I/R-induced myocardial tissue damage, no drugs that can abate myocardial I/R injury are being tested in clinical trials (3, 4). Therefore, the development of effective interventions and strategies to prevent myocardial I/R injury is of great clinical significance. Emerging evidence demonstrates that trimetazidine [1-(2, 3, 4-trimethoxybenzyl) piperazine dihydrochloride] is an agent with anti-ischemic properties that have been experimentally confirmed in various models, including cell culture, isolated organs, and *in vivo* (5-7). However, the mechanism that is responsible for trimetazidine-mediated cardioprotection against the pathogenesis of I/R injury remains unclear.

Hydrogen sulfide (H_2S), along with nitric oxide and carbon monoxide, is a well-recognized gasotransmitter capable of modulating numerous physiological processes (8). Endogenous generation of H_2S is mainly mediated by the enzyme cystathionine-γ-lyase (CSE) in the cardiovascular system (9). A growing body of evidence demonstrates that the CSE/H_2S pathway is part of a cardioprotective mechanism, playing a key role in *in vivo* and *in vitro* models of myocardial I/R disease (10, 11). In addition, a number of studies have revealed that H_2S mediates cardioprotection via the inhibition of myocardial inflammation, apoptosis,

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oxidative stress, and mitochondrial dysfunction in myocardial I/R injury, and that the promotion of H_2S generation and overexpression of CSE decrease the severity of the myocardial I/R injury (12-14). These findings indicate that enhancement of the CSE/H_2S pathway is beneficial in I/R injury treatment. However, it is not known whether the CSE/H_2S pathway is also involved in the cell-protective effect of trimetazidine against myocardial I/R injury.

To the best of our knowledge, this study is the first to examine the effects of trimetazidine on the CSE/H_2S pathway in hypoxia/reoxygenation (H/R)-treated H9c2 cells (an *in vitro* cell model of myocardial I/R injury). The aim was to determine whether the enhancement of the CSE/H_2S pathway, induced by trimetazidine, is a potential novel therapeutic approach to prevent myocardial I/R injury.

Methods

Cell culture

The embryonic rat heart-derived H9c2 cell line was purchased from the American Type Culture Collection (CRL1446; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. C11965500BT) supplemented with 10% (v/v) fetal bovine serum (cat. no. 10270-106) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 mg/ml penicillin/streptomycin (cat. no. ST488; Beyotime Institute of Biotechnology, Shanghai, China) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 2–3 days. The cells were sub-cultured or subjected to subsequent experimental procedures at 70%–80% confluence.

H/R injury model establishment and cell treatment

To establish an *in vitro* model of H/R injury, following cell growth at 70% confluence, the cell culture medium was changed to serum-free low-glucose DMEM and the cells were placed into a tri-gas incubator containing 94% N_2, 5% CO_2, and 1% O_2 (HF 100; Heal Force Bio-meditech Holdings, Ltd., Shanghai, China) for 6 h, which was treated as the hypoxia process. Subsequently, reoxygenation was initiated by incubating the cells in complete DMEM at 37°C with 5% CO_2 for 12 h. The cells in the control group were cultured under normoxic conditions. The cells were pretreated with trimetazidine (Servier Pharmaceutical, Co., Ltd, Tianjing, China) (0.1, 1, 10 or 100 μM) for 1 h and then exposed to the aforementioned H/R treatment to investigate the effect of trimetazidine on the H/R-induced H9c2 cells. To investigate the role of the CSE/H_2S pathway in the cardioprotection of trimetazidine, H9c2 cells were transfected with specific siRNA against human CSE (si-CSE; 50 nM) or scramble siRNA (si-scramble; 50 nM), and then treated with trimetazidine (10 μM) for 1 h prior to H/R treatment.

siRNA transfection

To knock down the CSE gene, H9c2 cells were transfected with si-CSE or si-scramble (both 50 nM; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine® 3000 (cat. no. L3000001; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocols. The related sequences were as follows: si-CSE sense, 5′-GGUUUAGCAGCCACUGUAAdTdT-3′; and antisense, 5′-UUACAGUGGCUGCUAAACCdTdT-3′; and si-scramble sense, 5′-UUCUCCGAACGUGUCACGUdTdT-3′; and antisense, 5′-ACGUGACACGUUCGGAGAAdTdT-3′. Following 6 h of transfection, the medium was replaced, and the silencing efficiency was determined by western blot analysis at 48 h after transfection.

Cell viability assay

The H9c2 cell viability was determined with a Cell Counting Kit-8 (CCK-8; cat. no. C0038; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. CCK-8 contains a yellow dye that is reduced by succinate dehydrogenase in the mitochondria of living cells to form soluble blue-purple formazan, whereas dead cells cannot perform this function. In brief, H9c2 cells were transferred to 96-well plates (3000 cells/well) and treated as discussed above. Subsequently, 10 μl CCK-8 solution was added to each well and incubated with the cells at 37°C for 4 h. The optical density value at the wavelength of 450 nm was measured with a multi-detection microplate reader (Victor2 1420; PerkinElmer, Inc., Waltham, MA, USA), and the results are presented as a percentage compared to the control group. All experiments were repeated at least three times.

Lactate dehydrogenase activity detection

The activity of lactate dehydrogenase (LDH) released from the cytosol of damaged cells into the supernatant, an indicator of cytotoxicity, was determined using the LDH cytotoxicity detection kit (cat. no. 11644793001; Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Briefly, following the aforementioned treatment of H9c2 cells, the culture supernatant from each sample was collected, and 100 μl supernatant was transferred to a 96-well plate and incubated with 100 μl freshly prepared reaction mixture for 30 min at 37°C. Following the addition of 50 μl stop solution, the absorbance at 490 nm was measured using a microplate spectrophotometer (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA).

H_2S content measurement

The levels of H_2S in H9c2 cells were determined by methylene blue, as described by Chunyu et al. (15) with modifications (16). In brief, cells were homogenized in ice-cold PBS, and the assay mixture (500 μl), including cell homogenate (450 μl), L-cysteine (10 mM; 20 μl), pyridoxal 5′-phosphate (2 mM; 20 μl), and saline (10 μl), was incubated at 37°C for 20 min in tightly sealed Eppendorf vials. Then, 1% zinc acetate (250 μl) was injected to trap formed H_2S, followed by the addition of 10% trichloroacetic acid (250 μl) to precipitate the protein and stop the reaction. Subsequently, N,N-dimethyl-p-phenylenediamine sulfate (20 mM; 133 μl) and $FeCl_3$ (30 mM; 133 μl) were added; and the mixture was

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incubated for 10 min. The absorbance (670 nm) of the resulting solution was measured using a 96-well microplate reader. The H_2S level was calculated using a standard curve plotted from 3.125–100 μM NaHS.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

Total RNA was extracted from cells using RNAsimple Total RNA kit (cat. no. DP419; Tiangen Biotech Co., Ltd., Beijing, China), following the manufacturer's protocol. The cDNA was prepared using the PrimeScript™ RT Master Mix kit (RR00036A, Takara, Osaka, Japan), and qPCR was carried out with the SYBR Green PCR Master Mix (RR036A, Takara, Osaka, Japan) using the ABI 7500 Real-time PCR System (ABI, Carlsbad, CA, USA), according to the manufacturers' instructions. The final volume of the PCR reaction mixture (20 μl) contained SYBR Premix Ex Taq II (10 μl), primers (1 μl each), cDNA (1 μl), and RNase-free H_2O (8 μl); and the cycling parameters for amplification were as follows: a denaturation step at 95˚C for 15 s, followed by 45 cycles at 95˚C for 10 s, 60˚C for 20 s, and 72˚C for 30 s. The GAPDH expression was used as the internal control. The primer sequences were as follows: CSE forward, 5′-GGCCTGGTGTCTGTTAATTGT-3′; and reverse, 5′-GCCATTCCGTTTTTGAAATGCT-3′; and GAPDH forward, 5′-TGACTTCAACAGCGACACCCA-3′; and reverse, 5′-CACCCTGTTGCTGTAGCCAAA-3′. Relative mRNA levels were calculated with the Data Assist Software version 3.0 (Applied Biosystems/Life Technologies) according to the $2^{-\Delta\Delta Ct}$ method.

Caspase-3 activity assay

The activity of caspase-3 was determined using a colorimetric assay kit (cat. no. 878-BC; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, H9c2 cells were lysed in the lysis buffer supplied in the kit, and equal amounts of protein were incubated with the reaction buffer including dithiothreitol as substrate for caspase-3 at 37˚C for 2 h in the dark. The absorbance at 405 nm was measured using a microplate reader (Thermo Scientific, New York City, NY, US). Each experiment was independently repeated three times.

Reactive oxygen species accumulation detection

The intracellular reactive oxygen species (ROS) production was determined with a 2',7'-dichlorofluorescin diacetate (DCFH-DA) ROS assay kit (cat. no. HY-D0940; Molecular Probes; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. The H9c2 cells were treated for a certain period of time, and then were harvested, washed, and resuspended in DCFH-DA solution (10 μM). The mixture was incubated at 37˚C for 20 min in a dark room and the results were analyzed by an FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using a 488 nm excitation filter and a 525 nm emission filter. The data are expressed as percentage of fluorescence intensity relative to the control cells. A fluorescence microscope (Olympus Corporation, Tokyo, Japan) captured the images.

Lipid peroxidation marker (MDA) content measurement

The MDA content was determined using the MDA assay kit (Bioxytech LPO-586; GT Biopharma, Westlake Village, CA, USA) following the manufacturer's protocol. Following the aforementioned treatment, the cells were washed with PBS, harvested by scrapping in ice-cold PBS, and then centrifuged at 3000 x g for 10 min at 4˚C to remove cell debris. The MDA content in the supernatant was measured by recording the absorbance at 586 nm using the Epoch 2 spectrophotometer. Total MDA levels (nM) were calculated based on a standard curve and normalized to total protein levels.

Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity determination

Following the aforementioned treatment for 24 h, H9c2 cells were harvested, homogenized, and centrifuged at 10,000 x g for 12 min. The lysates were stored at −70˚C until they were required for the enzyme activity assay. The SOD activity in the H9c2 cells was measured using an SOD assay kit (cat. no. S311; Dojindo Molecular Technologies, Inc.). GSH-Px activity in H9c2 cells was measured using a colorimetric assay kit (cat. no. E20130114019; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Both assays were performed according to the protocols of the manufacturers of the kits.

Western blot analysis

Following the aforementioned treatment for 24 h, the protein from the H9c2 cells in the different experimental groups was extracted using radioimmunoprecipitation assay buffer supplemented with complete protease inhibitor cocktail (cat. no. 11836170001; Roche Applied Science) at 4˚C. The mixture was incubated for 30 min, and the cell lysates were centrifuged at 14,000 x g for 10 min at 4˚C. The amount of protein was determined using the Pierce BCA protein assay (cat. no. 23229; Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membrane (cat. no. ISEQ00010; Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% non-fat milk for 2 h at room temperature, and incubated overnight at 4˚C with the following primary antibodies: anti-apoptosis regulator BAX (cat. no. ab32503), anti-apoptosis regulator Bcl-2 (cat. no. ab32124) (both rabbit monoclonal; 1:1,000; Abcam, Cambridge, UK), anti-CSE (cat no. 30068), and anti-GAPDH (cat. no. 5174) (both rabbit polyclonal; 1:2,000; Cell Signaling Technology Europe, B.V., Leiden, The Netherlands). Following washing with Tris-buffered saline containing Tween-20 three times (5 min each), the membrane was incubated at 37˚C for 2 h with horseradish-peroxidase-conjugated anti-rabbit (cat. no. 323-005-024) or anti-mouse (cat. no. 223-005-024) IgG (both 1:40,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The protein bands were visualized using the Pierce enhanced chemiluminescence western blot substrate (cat. no. 32134; Thermo Fisher Scientific, Inc.) and quantified as a ratio to GAPDH using the Quantity One software 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Data are presented as the mean±standard error of the mean from three independent experiments. The normal distribution of the data was evaluated by the Shapiro–Wilk tests. The Tukey test was applied for comparing of group means. Statistical significance was determined using one-way analysis of variance followed by the variance homogeneity test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Trimetazidine increases cell viability and decreases LDH activity in H/R-treated H9c2 cells. To determine the trimetazidine-induced protection of H9c2 cells against injury following H/R treatment, the cell viability and LDH activity were detected. As observed in Figure 1, the cell viability (Fig. 1a) and LDH activity were significantly lower (Fig. 1b), in the H/R-treated group compared with the control. However, trimetazidine treatment notably reversed these effects, in particular at 10 μM. Therefore, 10 μM trimetazidine was the concentration of choice in the subsequent experiments. These results suggest that trimetazidine protects H9c2 cells against H/R-induced injury.

Trimetazidine blocks the H/R-induced inhibition of the CSE/H_2S pathway in H9c2 cells. To determine whether the CSE/H_2S pathway is involved in the protective effects of trimetazidine against myocardial H/R injury, we investigated the effects of trimetazidine on the levels of H_2S and CSE. As demonstrated in Figure 2, H/R treatment markedly decreased the H_2S level in H9c2 cells, whereas trimetazidine pretreatment mitigated this effect (Fig. 2a). The results from the RT-qPCR and western blot analyses revealed that compared with the control cells, CSE mRNA (Fig. 2b), and protein (Fig. 2c, 2d) levels, respectively, were significantly downregulated in the H/R-treated cells. However, these effects were also avoided by trimetazidine pretreatment. These results indicate that trimetazidine enhances the CSE/H_2S pathway under H/R conditions in H9c2 cells.

CSE knockdown attenuates trimetazidine-induced protection of H9c2 cells against H/R-induced injury. To further confirm the role of the CSE/H_2S pathway in the cardioprotective action of trimetazidine, H9c2 cells were transfected with si-CSE to induce CSE knockdown. As expected, the western blot analysis results revealed that the expression of the CSE protein was suppressed following si-CSE transfection (Fig. 3a, 3b). Subsequently, it was demonstrated that the CSE knockdown induced by si-CSE transfection notably inhibited the trimetazidine-induced increase in cell viability (Fig. 3c) and decrease in LDH activity (Fig. 3d) in H/R-treated H9c2 cells, whereas transfection with si-CSE or si-scramble alone did not affect cell viability or LDH activity. These results suggest that the CSE/H_2S pathway mediates the inhibitory effect of trimetazidine on the H/R-induced toxicity in H9c2 cells.

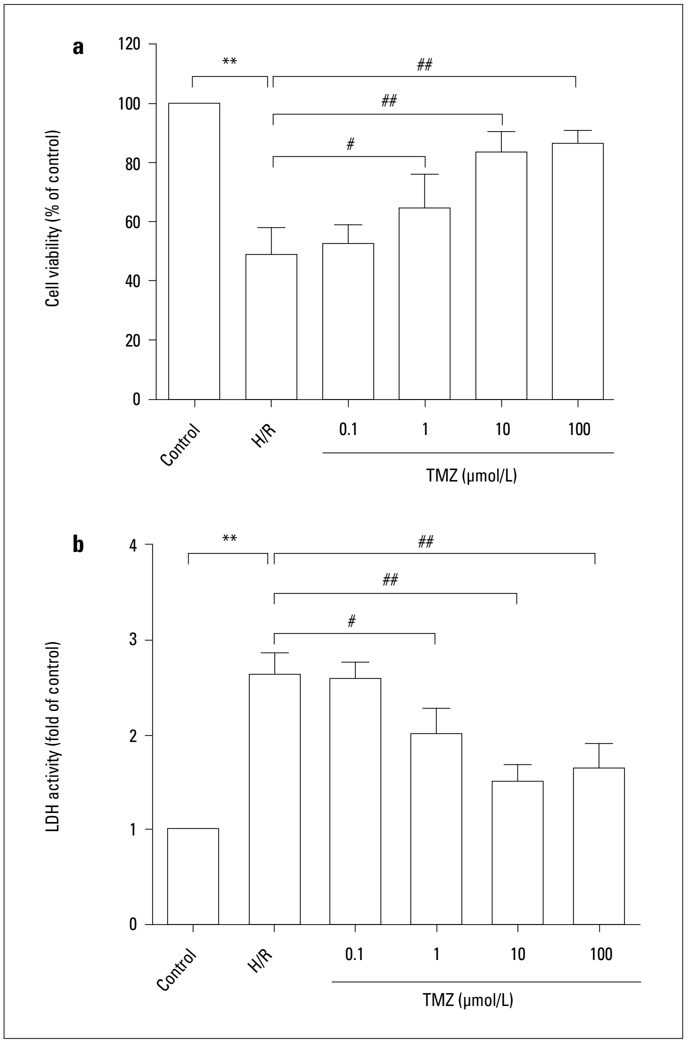

Figure 1. Effects of trimetazidine on cell viability and LDH activity in H/R-treated H9c2 cells. (a) Cell Counting Kit-8 assay results of H9c2 cells exposed to trimetazidine (0.1, 1, 10, or 100 μM) followed by H/R treatment. (b) LDH activity in the cell supernatant. The values represent the mean±standard error of the mean (n=3). ***P*<0.01 vs. control group; #*P*<0.05 and ##*P*<0.01 vs. H/R treatment group.

LDH - lactate dehydrogenase; H/R - hypoxia/reoxygenation; TMZ – trimetazidine

CSE knockdown alleviates the trimetazidine-induced decrease of apoptosis in H/R-treated H9c2 cells. The apoptosis of cardiomyocytes is another important indicator of myocardial I/R injury (17). To determine whether trimetazidine protects against cardiomyocyte apoptosis through the CSE/H_2S pathway, we investigated the effects of CSE knockdown on apoptosis. Caspase-3 is an important factor in cell apoptosis (18), and BAX and Bcl-2 are pro- and anti-apoptotic proteins, respectively (19). Trimetazidine mitigated the H/R-induced upregulation of caspase-3 activity in H9c2 cells, while this effect was reversed by si-CSE transfection (Fig. 4a). Western blot analysis (Fig. 4b) demonstrated that trimetazidine markedly decreased BAX expression (Fig. 4c) and increased Bcl-2 expression (Fig. 4d) in H/R-treated H9c2 cells. However, these effects of trimetazidine were mitigated by si-CSE. These results suggest that the CSE/

Anatol J Cardiol 2019; 22: 102-11
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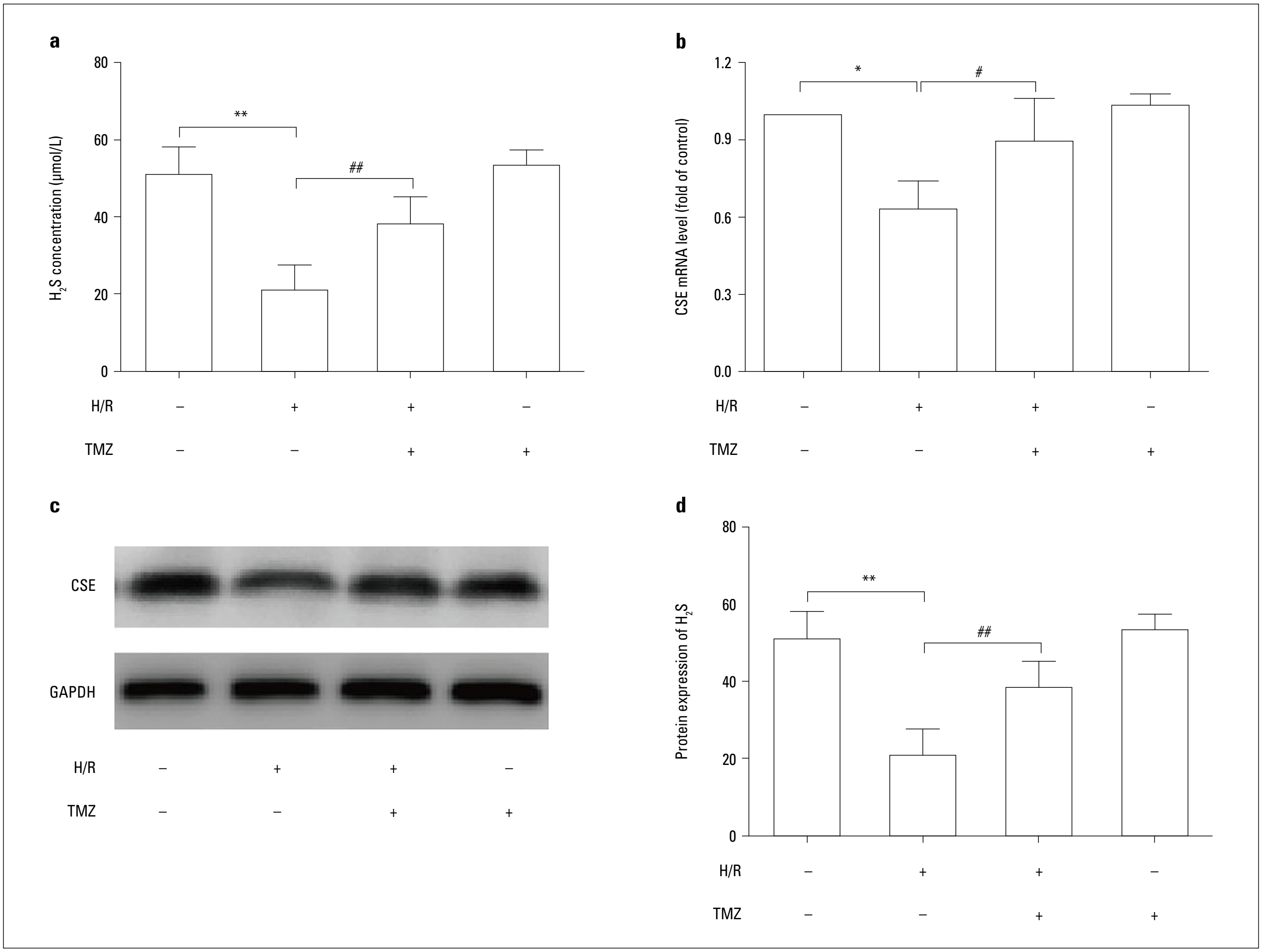

Figure 2. Effects of trimetazidine on the CSE/H_2S pathway in H/R-treated H9c2 cells. H9c2 cells were exposed to trimetazidine (10 μM) for 1 h followed by H/R treatment. (a) Measurement of the H_2S level in the cell culture supernatant by spectrophotometry with slight modifications using L-cysteine at various concentrations as a substrate. (b) The relative level of CSE mRNA determined by reverse transcription-quantitative polymerase chain reaction. (c) Western blot analysis of the CSE protein expression (d). The values represent the mean±standard error of the mean (n=3). **P*<0.05 and ***P*<0.01 vs. control group; and #*P*<0.05 and ##*P*<0.01 vs. H/R treatment group.
CSE - cystathionine γ-lyase; H_2S - hydrogen sulfide; H/R - hypoxia/reoxygenation

H_2S pathway contributes to the trimetazidine protection of H9c2 cells against H/R-induced apoptosis.

CSE knockdown alleviates the trimetazidine-induced decrease in oxidative stress in H/R-treated H9c2 cells. Oxidative stress serves a major role in myocardial I/R pathology, and the release of intracellular ROS is known as a mediator of the intracellular signaling cascade, which can trigger a series of events, including apoptosis (20). Therefore, we investigated the effects of CSE knockdown on oxidative stress under H/R conditions in trimetazidine-treated H9c2 cells. As observed in Figure 5, trimetazidine markedly attenuated the overproduction of ROS in H9c2 cells, as indicated by the decrease in green fluorescence, while this effect was eliminated by si-CSE transfection (Fig. 5a, 5b). si-CSE also led to the reversal of the trimetazidine-induced decrease in MDA in H/R-treated H9c2 cells (Fig. 5c). In addition, CSE knockdown overturned the trimetazidine-induced downregulation of NADPH oxidase 2 (Nox2) protein in H/R-treated H9c2 cells (Fig. 5d, 5e). Furthermore, transfection with si-CSE depleted the trimetazidine-induced antioxidant defenses as evidenced by the diminished SOD activity (Fig. 5f) and GSH-Px (Fig. 5g) level, as compared to trimetazidine and H/R co-treatment group. These results indicated that CSE/H_2S pathway mediates trimetazidine-induced the attenuation of oxidative injury in H/R-treated H9c2 cells.

Discussion

In this study, the signaling pathways by which trimetazidine triggers anti-apoptosis and antioxidant outcomes in H/R-induced myocardial injury were demonstrated. Specifically, the study fo-

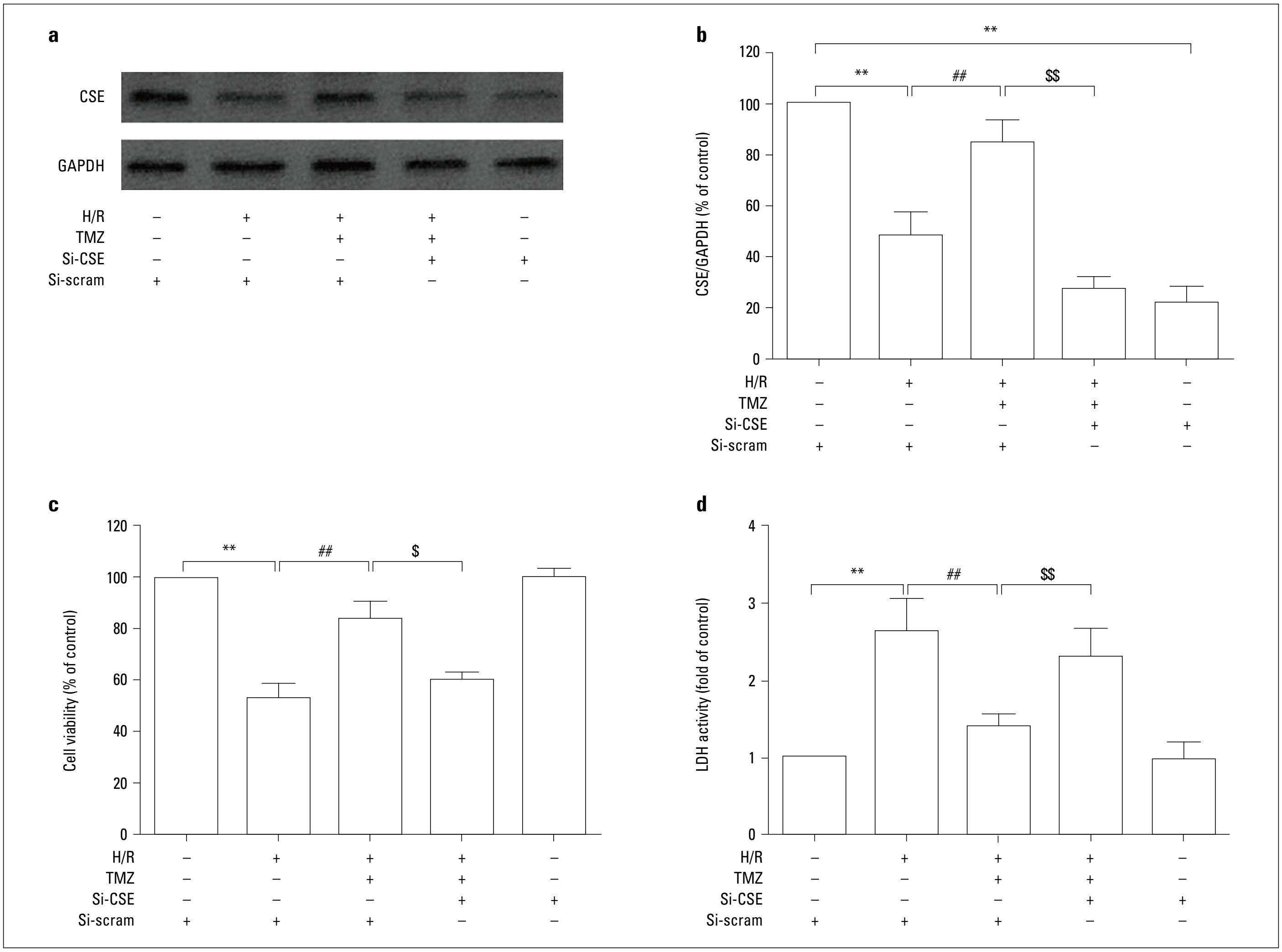

Figure 3. Effects of the CSE knockdown induced by transfection with si-CSE on the trimetazidine-induced protection of H9c2 cells against H/R-induced injury. H9c2 cells were transfected with si-CSE or si-scramble and exposed to trimetazidine (10 μM) for 1 h prior to H/R treatment. (a) Determination of CSE protein expression by western blot analysis, reflecting the transfection efficiency of si-CSE. (b) Quantitative analysis of the western blotting result. (c) Measurement of the viability of H9c2 cells by the Cell Counting Kit-8 assay. (d) Detection of LDH activity in the cell supernatant by the LDH cytotoxicity detection kit. The values represent the mean±standard error of the mean (n=3). $^{**}P<0.01$ vs. control group; $^{\#\#}P<0.01$ vs. H/R treatment group; $^{\$}P<0.05$ and $^{\$\$}P<0.01$ vs. trimetazidine and H/R co-treatment group

CSE - cystathionine γ-lyase; si-CSE - specific siRNA against human CSE; si-scram/si-scramble - scrambled siRNA; H/R - hypoxia/reoxygenation; LDH - lactate dehydrogenase

cused on the potential role of the CSE/H_2S system in these processes. It was found that trimetazidine significantly increased the cell viability and decreased LDH release in H/R-treated H9c2 cells. Additionally, trimetazidine increased the H_2S levels and the CSE mRNA and protein levels, promoting the CSE/H_2S pathway under H/R conditions. Overall, the major findings of this study on H9c2 cells are as follows: i) trimetazidine prevents against H/R-induced injury; ii) trimetazidine enhances the CSE/H_2S pathway under H/R conditions; iii) the CSE/H_2S pathway mediates trimetazidine-induced protection against H/R-induced injury; and iv) the CSE/H_2S pathway contributes toward the trimetazidine-induced inhibition of apoptosis and oxidative stress stimulated by H/R. These results confirmed for the first time that the enhancement of the CSE/H_2S system mediates the trimetazidine-induced protection of H9c2 cells against H/R injury, expanding our knowledge and understanding of the mechanism of action of trimetazidine and the role of the CSE/H_2S pathway in the protection against myocardial I/R injury.

Trimetazidine, a metabolic agent with several properties, is included in the current European Society of Cardiology 2013 guidelines on the management of coronary heart disease. A growing body of evidence supports the theory that trimetazidine exerts significant protective effects against myocardial I/R injury (21-23). In accordance with these findings, this study revealed that trimetazidine notably eliminated H/R-induced cardiomyocyte damage, improving cell viability and LDH activity, reducing the indicators of cell injury (24), and confirming the protective function of trimetazidine against cardiac H/R-induced injury.

It was reported that TMZ protected HF ventricular myocytes from cytosolic Ca (2+) overload and subsequent hypercontrac-

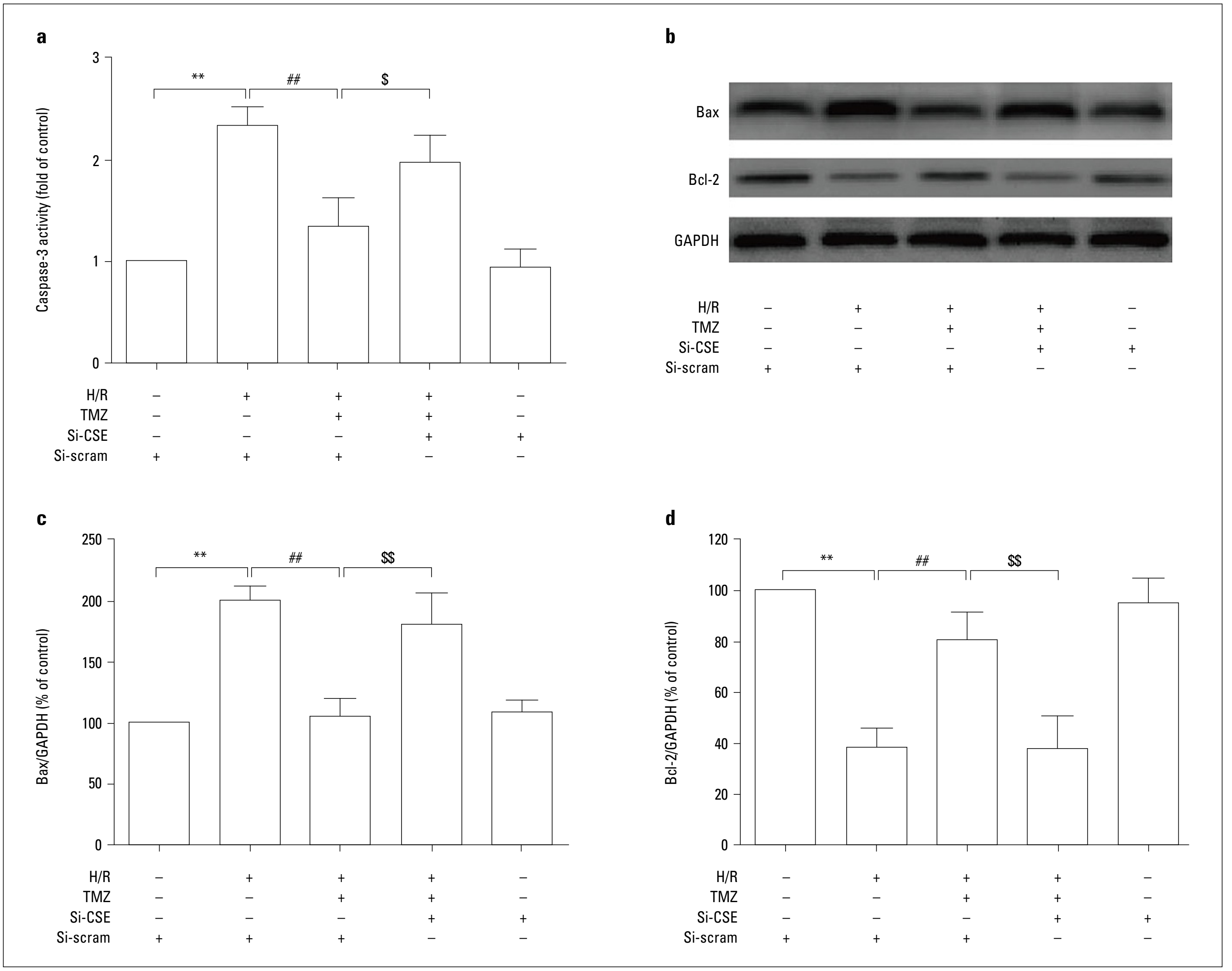

Figure 4. Effects of the CSE knockdown on the trimetazidine-induced inhibition of apoptosis in H/R-treated H9c2 cells. H9c2 cells were transfected with si-CSE or si-scramble and exposed to trimetazidine (10 μM) for 1 h prior to H/R exposure. (a) Analysis of the caspase-3 activity by a colorimetric assay kit. (b) Western blot analysis and quantification of the expression of apoptosis regulators (c) BAX and (d) Bcl-2 in H9c2 cells. The values represent the mean±standard error of the mean (n=3). $^{**}P<0.01$ vs. control group; $^{\#\#}P<0.01$ vs. H/R treatment group; $^{\$}P<0.05$ and $^{\$\$}P<0.01$ vs. trimetazidine and H/R co-treatment group

CSE - cystathionine γ-lyase; H/R - hypoxia/reoxygenation

ture, induced by electrical and ß-adrenergic (isoproterenol) stimulation (25). This effect was mediated by the ability of TMZ to protect HF myocytes against mitochondrial permeability transition pore (mPTP) opening via attenuation of ROS generation by the mitochondrial electron transport chain and uncoupled mitochondrial nitric oxide synthase (mtNOS) (25). Another study has found that H_2S could ameliorate cardiac function (26). Treatment with NaHS inhibited the occurrence of cardiac apoptosis and improved cardiac structure. H_2S reduced the expression of the cleaved caspase-3, NOX4, and the leakage of cyt c from the mitochondria to the cytoplasm. Exogenous H_2S could maintain the mitochondrial membrane potential and reduce ROS production in the mitochondria. Therefore, H_2S reduces oxidative stress due to cardiac hypertrophy through the cardiac mitochondrial pathway (26). TMZ-induced enhancement of autophagy was considered to be related to increased AMP-activated protein kinase (AMPK) phosphorylation and decreased mammalian target of rapamycin (mTOR) phosphorylation (21).

Several studies have demonstrated that the CSE/H_2S pathway plays a cardioprotective role in myocardial I/R injury via reducing the extent of ischemic infarction, promoting cell survival, and decreasing cardiomyocyte apoptosis, whereas inhibition of this pathway contributes to the process of myocardial I/R injury (27, 28). The results of this study revealed that H/R treatment notably decreases H_2S generation and CSE protein expression in H9c2 cells, which demonstrates the inhibitory effects of H/R injury on the CSE/H_2S pathway, in line with the study by Salloum et al. (14), which reported that endogenous CSE/H_2S system was inhibited

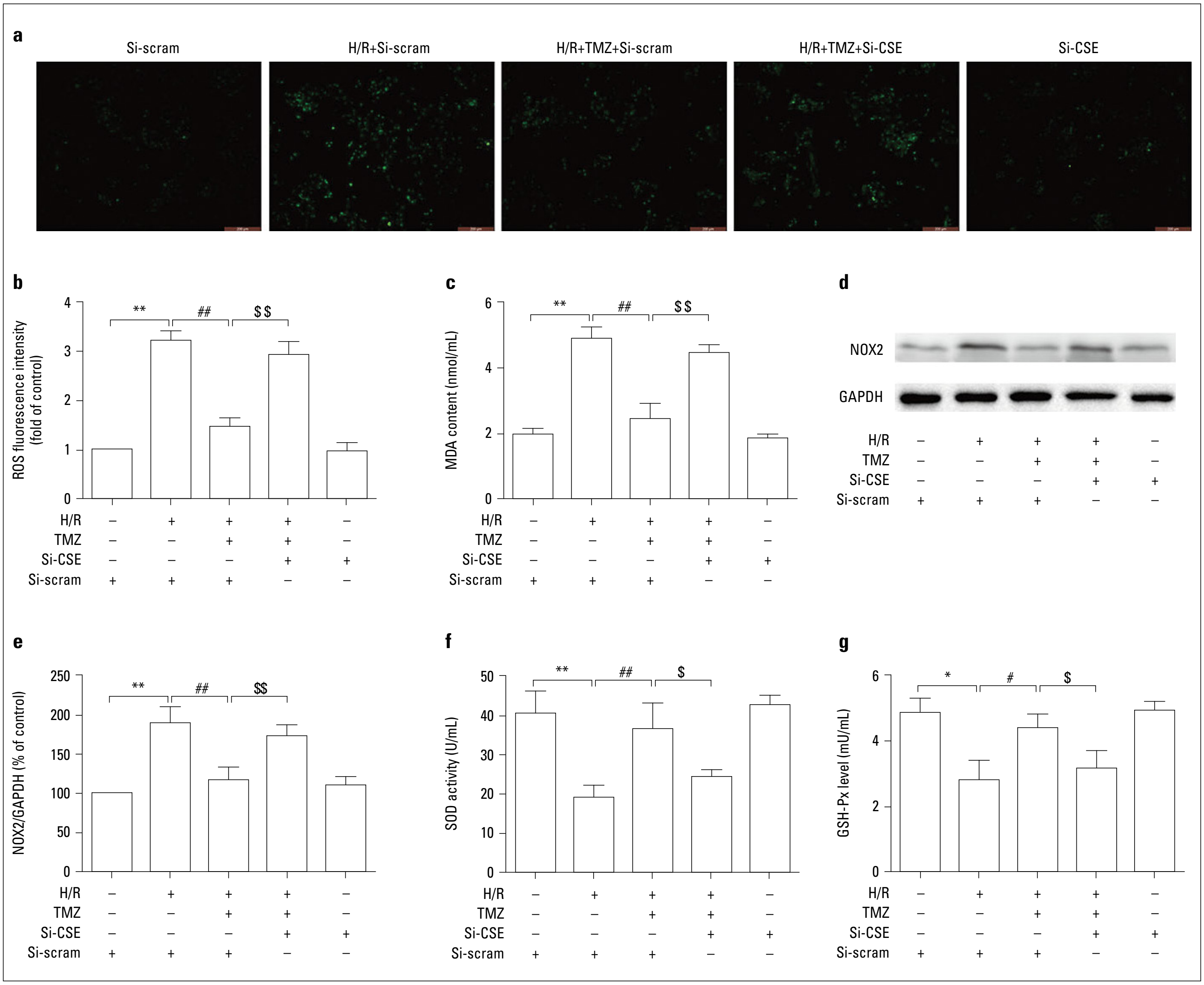

Figure 5. Effects of CSE knockdown on oxidative stress in the presence or absence of trimetazidine in H/R-treated H9c2 cells. H9c2 cells were transfected with si-CSE or si-scramble and exposed to trimetazidine (10 μM) for 1 h prior to H/R treatment. (a) Determination of intracellular ROS production using probe 2′,7′-dichlorofluorescin diacetate. The green fluorescence in the cells was observed using a fluorescent microscope (x200). (b) Quantitative analysis of ROS production was carried out using an FACS Calibur flow cytometer. (c) Measurement of MDA content in H9c2 cells using a lipid peroxidation assay kit. (d) The expression of Nox2 protein by western blot analysis. (E) Quantitative analysis of Nox2 protein expression. (f) Detection of SOD activity using an SOD assay kit. One unit of SOD is defined as the amount of enzyme in protein sample solution (20 μl) that inhibits the reduction of WST-1 by superoxide anions by 50%. (g) Detection of GSH-Px level using a GSH-Px assay kit (colorimetric method). The values represent the mean±standard error of the mean (n=3). **P*<0.05 and ***P*<0.01 vs. control group; #*P*<0.05 and ##*P*<0.01 vs. H/R treatment group; $*P*<0.05 and $$*P*<0.01 vs. trimetazidine and H/R co-treatment group.

CSE - cystathionine γ-lyase; H/R - hypoxia/reoxygenation; ROS - reactive oxygen species; MDA - lipid peroxidation marker; Nox2 - NADPH oxidase 2; SOD - superoxide dismutase; GSH-Px - glutathione peroxidase

in cardiac I/R injury. Additionally, certain studies have revealed the importance of the CSE/H_2S pathway in the cardioprotective mechanism of a number of pharmacological or natural compounds, including chelerythrine (12), zofenopril (13), and beetroot juice (14). However, the role of the CSE/H_2S axis in the function of trimetazidine has not yet been reported. To the best of our knowledge, this study is the first to demonstrate that trimetazidine pretreatment markedly promotes H_2S production and CSE expression. The inhibition of the CSE/H_2S pathway, caused by si-CSE transfection, reversed the trimetazidine-induced protection of H9c2 cells against injury triggered by H/R. These results suggest that the CSE/H_2S pathway mediates the protective effect of trimetazidine against myocardial I/R injury.

Apoptosis is a genetically programmed form of cell death. Several studies have demonstrated that trimetazidine protects against cardiac I/R injury by decreasing cardiomyocyte apoptosis (5, 29). In this study, trimetazidine pretreatment decreased caspase-3 activity, the most important apoptotic factor, in H/R-

Anatol J Cardiol 2019; 22: 102-11
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treated H9c2 cells. Anti-apoptotic protein Bcl-2 and pro-apoptotic protein BAX play important pathophysiological roles in myocyte apoptosis following I/R injury (30). This study further revealed that trimetazidine decreased the expression of BAX and increased the expression of Bcl-2, in agreement with previous studies where trimetazidine was found to act protectively against myocardial I/R injury with a lower apoptotic cell death rate (5, 31). Notably, there is accumulating evidence that H_2S inhibits the apoptosis of cardiomyocytes induced by myocardial I/R injury, by regulating the Bcl-2/BAX ratio and caspase-3 activity in the myocardium (32, 33). In line with these findings, this study found that the inhibition of the CSE/H_2S pathway, induced by si-CSE, blocked the anti-apoptotic effect of trimetazidine during H/R injury. These results suggest that the CSE/H_2S pathway is involved in the trimetazidine-induced inhibition of apoptosis in myocardial H/R injury.

Emerging evidence has revealed that oxidative stress causes I/R injury, and excessive ROS production and an oxidant/antioxidant imbalance have long been recognized as major mediators of I/R injury (20). There are reports that H_2S exerts cardioprotective effects during I/R injury through enhancing antioxidant enzymes, including SOD and GSH-Px, to scavenge ROS, ultimately resulting in increased cell survival. Accumulating evidence indicates the curative effect of trimetazidine on the oxidative damage associated with myocardial H/R injury (34, 35). However, the role of the CSE/H_2S pathway in the protection of trimetazidine against oxidative stress-mediated injury remains unreported. This study is the first in which trimetazidine significantly reversed the H/R-induced increase of ROS and MDA, which is the end product of lipid peroxidation, and the decrease in the activity of antioxidant enzymes SOD and GSH-Px. Furthermore, these changes were markedly attenuated by the inhibition of the CSE/H_2S pathway by si-CSE. These results indicate that trimetazidine attenuates the myocardial damage caused by oxidative stress by activating the CSE/H_2S pathway.

There were also some limitations in this study. First, our study was performed only *in vitro* experiment. Further study with *in vivo* experiment is needed. Second, the mechanism investigated in this study was still not comprehensive. Further study involving the mechanism of the effect of trimetazidine is needed.

Conclusion

In conclusion, this study confirmed that trimetazidine acts protectively against cardiac I/R injury *in vitro*. The findings highlight the contribution of the CSE/H_2S pathway to this process via inhibiting apoptosis and oxidative stress. As no drugs that can abate myocardial I/R injury are being tested in clinical trials, the results of this study may provide a basis for the use of trimetazidine in the treatment of cardiac I/R injury in further clinical trial.

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